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Communication

[*Comunicação*]

A retrospective PCR investigation of avian *Orthoreovirus*, chicken infectious anemia and fowl *Aviadenovirus* genomes contamination in commercial poultry vaccines in Brazil

[*Estudo retrospectivo de vacinas avícolas vivas por PCR para os vírus da anemia das galinhas,* Aviadenovirus *aviário e* Orthoreovirus *aviário*]

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Commercial vaccines have been a major tool for inducing flock protection on the prevention of important infectious diseases of chickens. However, live vaccines may represent a risk, especially considering purity, by the presence of otherwise unknown infectious agents, therefore not searched for. Monitoring SPF flocks, source of eggs and tissues, based on low sensitivity methods, may have also contributed to risk. Fowl *Aviadenovirus* (FAdV), avian *Orthoreovirus* (ARV) and chicken anemia virus *Gyrovirus* (CAV) are major pathogens which had been present sub-clinically in specific pathogen free (SPF) flocks of chickens (Yuasa *et al.*, 1979; Cardona *et al*., 2000; Miller *et al*., 2001) and may be horizontally transmitted, on failed biosecurity, and vertically to the embryos (Schat, 2003). CAV was previously described in industrial (Brentano *et al.*, 1991) and free-range chickens (Barrios *et al*., 2009) in Brazil. SPF flocks are used in research and for the production of vaccines, and their infection may represent a most relevant epidemiological risk, especially concerning lack of purity of live vaccines, therefore requiring highly sensitive and specific monitoring assays.

A retrospective study was conducted with 26 freeze-dried live virus and two inactivated vaccines commercially available for poultry in Brazil, produced from 1991 to 2005, aiming to investigate the contamination with ARV, CAV and FAdV as extraneous genomes. Vaccines were available originally sealed and stored at refrigeration (4-8°C). Reference DNA for CAV was obtained from a vaccine strain (AviPro Thymovac®, Lohmann Animal Health, Germany), for *Aviadenovirus* from FAdV Phelps, kindly provided by Dr. Jane K. A. Cook, and RNA for ARV from the S1133 vaccine strain (Poulvac Reo, Fort Dodge). DNA extractions were performed using silica (Silicon dioxide, Sigma-Aldrich, USA) adsorption protocol previously described (Boom *et al*., 1999), removing protein with sodium iodide (Caxito *et al*., 2006). Silica adsorbed DNA was eluted by adding 50µl TE (5mM Tris-HCl pH 8.0, 0.5mM EDTA pH 8.0) and stored at -20ºC until tested. RNA extraction (Trizol®, Invitrogen, USA) was based on a protocol designed for infectious bursal disease virus (IBDV) (Gomes *et al*., 2005). All DNA and RNA extracts concentrations were estimated (NanoDrop ND-1000) for assay. The quantified RNA was immediately transcribed (300ηg) into ARV cDNA using reverse transcriptase (M-MLV, Promega, USA) according to the manufacturer instructions and using the external forward primer. As negative controls, the mixture of all reagents except for the DNA template were used. Total DNA was employed as template for CAV or FAdV PCR, or cDNA for ARV RT-PCR, using primers and protocols as previously described (Table 1) in a thermocycler (Maxygene, Axygen, USA). PCR products were resolved in agarose (1%) gel electrophoresis

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(TBE 0.5X - 100mM Tris-base pH 8.3, 25 mM EDTA and 50mM boric acid) and expected molecular sizes (Table 1) visualized at UV transiluminator (Hoefer Scientific Instruments, USA).

Results indicated that vaccines from three different manufacturers (A, B and C) presented the contaminating genomes (Table 2). Out of the 26 live vaccines tested, five (19%) presented the expected CAV products, with molecular sizes of 388 and 211bp, for the first and nested reactions, respectively. Nine (34.6%) vaccines presented the expected (738 and 342bp) amplicons for ARV, for the first and nested reactions, respectively. Three (11%) vaccines showed the expected 897bp amplicon for FAdV. The Newcastle disease (La Sota strain) vaccine, produced in Jan/1991 and May/1992, and the avian encephalomyelitis (Van Roekel strain) vaccine, produced in Jan/1994, were all from manufacturer A and contained the three specific amplicons (CAV, ARV and FAdV) contaminations. Two Marek's disease HVT lyophilized vaccines, from two different manufacturers (B and C), produced in February and July/1996, presented both CAV and ARV genome contaminations. One Newcastle disease (Jan/1998), one infectious bursal disease (Nov-Dec/1998) and two infectious bronchitis (Sept-Dec/1998) vaccines, all from the same manufacturer (B), presented ARV contamination.

More recently manufactured vaccines, produced in 2001 and onwards, were tested negative for genomes of the three viruses.

It was refreshing to know that vaccines produced from 2001 and onwards were free from the contaminating genomes, indicating improved quality of the specific pathogen free (SPF) flocks. Vaccines of manufacturers D, E, F, G and H have never shown contamination. Although not considered for discussion, due to the small sample, two commercial inactivated oil-emulsion vaccines were tested negative for all genomes here studied.

Table 1. Primers and amplicons for *Aviadenovirus*, avian *Orthoreovirus* and chicken infectious anemia virus

PCR	Primer	Product (bp)
Aviadenovirus Meulemans *et al*. (2001).	F: 5'-(CAA GTT CAG GCA GAC GGT)-3'	
	R: 5'- (TAG TGA TGC CGC GAC ATC AT)-3'	897
Avian *Orthoreovirus* Liu *et al*. (1997)	Transcription 5'-(ATT GAA TTC TCT GTT ATC TCA ACC TTG)-3'	
	External S1C 5-(ATT GAA TTC TCT GTT ATC TCA ACC TTG)-3'	
	External S1D 5'-(AAG GAA TTC GTT GAG AAC AGA AGT AGG)-3'	738
	Internal S1E 5-(TCT GAA TTC ATC GCA GCG AAG AGA GGT CG)-3'	
	Internal S1F 5'-(AGT GAA TTC AGT ATC GCC GCG TGC GCA G)-3'	342
Chicken infectious anemia virus	External O3F: 5'-(CAA GTA ATT TCA AAT GAA CG)-3'	
Cardona *et al*. (2000).	External O3R: 3'-(TTG CCA TCT TAC AGT CTT AT)	388
	Internal N3: 5'-(CCA CCC GGA CCA TCA AC)-3'	
	Internal N4: 3'-(GGT CCT CAA GTC CGG CAC ATT C)	211

Table 2. PCR investigation of ARV, CAV and FAdV in commercial poultry vaccines

Month/Year[1]	Vaccine[2]	Manufacturer[3]	ARV[4]	CAV[5]	FAdV[6]
1. Jan/1991	Newcastle disease	A	+	+	+
2. Dec/1991	Infectious bronchitis	C	-	-	-
3. May/1992	Newcastle diasease	A	+	+	+
4. Dec/1992	Infectious bronchitis	B	-	-	-
5. Jan/1994	Avian encephalomyelitis	A	+	+	+
6. Jan/1995	Fowl pox	E	ND	ND[7]	-
7. Jan/1995	Infectious bronchitis	H	ND	-	ND
8. Feb/1996	Marek's disease	B	+	+	-
9. Jul/1996	Marek's disease	C	+	+	-
10. Sep/1997	Infectious bronchitis	B	-	-	-
11. Jan/1998	Newcastle disease	B	+	ND	ND
12. Sep/1998	Infectious bronchitis	B	+	-	-
13. Nov/1998	Infectious bursal disease	B	-	-	-
14. Dec/1998	Infectious bursal disease	B	+	-	-
15. Dec/1998	Infectious bronchitis	B	+	-	-
16. Sep/2001	Infectious bronchitis	F	-	-	-
17. Feb/2002	Infectious bronchitis	D	-	-	-
18. May/2004	Egg drop syndrome[8]	C	-	-	-
19. Jul/2004	Infectious bursal disease	C	-	-	-
20. Jul/2004	Newcastle disease	C	ND	-	-
21. Aug/2004	Infectious bronchitis	C	-	-	-
22. Nov/2004	Newcastle disease	D	-	-	-
23. Mar/2005	Infectious bronchitis	D	-	-	-
24. May/2005	Avian encephalomyelitis	C	-	-	-
25. Jun/2005	Newcastle disease	E	-	-	-
26. Aug/2005	Newcastle disease	D	-	-	-
27. Aug/2005	Fowl pox and Avian encephalomyelitis	G	-	-	-
28. Sep/2005	Infectious bronchitis/Infectious Coryza/Egg drop syndrome[8]	D	-	-	-

Notes: 1. Date of manufacturing; 2. Live lyophilized; 3. Major manufacturers in Brazil; 4. ARV: Avian *Orthoreovirus*; 5. CAV: Chicken anemia vírus *Gyrovirus*; 6. FAdV: Fowl *Aviadenovirus*; 7. ND: not done; 8. Inactivated vaccine; +: Positive for specific amplicon; -: Negative for specific amplicon.

It is hypothesized that the detection of contaminating virus genomes (ARV, CAV and FAdV) may indicate a risk for extraneous viral activity in such vaccines, especially taking into consideration the highly resistant nature of the evaluated viruses and the type of vaccine (live), formulated to preserve virus infectivity, for inducing infection and immunity.

It is of obviously ultimate importance the purity and innocuity of live vaccines, quality dependent on the health status of SPF flocks and eggs. However, a few methods employed for monitoring the investigated infections in SPF flocks may be of low sensitivity, as the agar gel immunodiffusion for FAdV (Fadly *et al*., 1980; Mockett and Cook, 1983), presently at use for such flocks. Also, monitoring CAV infection based on antibody detection may present false negative results for low virus excreting flocks (Miller *et al*., 2001). ARV tests for SPF flocks are also based on antibody detection. However, vaccine contamination may principally occur due to SPF flock breakdowns, due to inadequate biosecurity standards or a recent infection prior to egg collection, than to insufficient sampling and testing of the flock or inadequate sensitivity of used tests. The mass administration of a contaminated live vaccine could enable rapid national spread of an infectious agent, of difficult subsequent eradication (Contingency, 2006). PCR methodology enables highly specific and sensitive detection of genomes of live and inactivated agents. Such advances are becoming

available and being recommended for the rapid and specific diagnosis of relevant viruses, bacteria and other infectious agents of disease (World, 2008). In view of the risk and in order to ensuring rapidity, high sensitivity and high specificity for monitoring SPF flocks, the use of molecular methods may be valuable.

Keywords: Chicken, vaccine, avian *Orthoreovirus, Aviadenovirus, Gyrovirus*

RESUMO

*Vacinas avícolas vivas comerciais produzidas entre 1991 e 2005 foram examinadas para a presença de genomas dos vírus da anemia infecciosa das galinhas (*Gyrovirus *CAV), da hepatite por corpúsculo de inclusão* (Aviadenovirus *FAdV) e da artrite viral/síndrome da má absorção* (Orthoreovirus *aviário ARV). Vinte e seis partidas de vacinas vivas liofilizadas de oito fabricantes com lacre original foram examinadas. As extrações de DNA e PCR de CAV e FAdV, e de RNA e RT-PCR para ARV, foram descritas previamente. Contaminações triplas de ARV, CAV e FAdV foram detectadas em vacinas de mesmo fabricante, produzidas em 1991 e 1992 contra a doença de Newcastle (DN), e para a encefalomielite aviária, produzida em 1994. ARV e CAV em co-infecção foram encontrados em vacinas contra a doença de Marek liofilizadas produzidas em 1996 por dois fabricantes diferentes. Genoma de ARV foi detectado em vacinas contra a bronquite infecciosa de setembro e dezembro de 1998, doença infecciosa bursal, de dezembro de 1998 e DN de janeiro de 1998. Três dos oito fabricantes apresentaram vacinas com contaminação e cinco nunca apresentaram vacinas contaminadas. Nenhuma vacina produzida a partir de 2001 apresentou contaminação. Cogita-se um papel epidemiológico para vacinas vivas, como fonte de infecção para ARV, CAV e FAdV e, potencialmente determinante da atual alta disseminação destes.*

Palavras-chave: galinha, vacina, Aviadenovirus, Gyrovirus, Orthoreovirus

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